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Photosensitizer-induced cross-linking: a novel approach for improvement of physicochemical and structural properties of gelatin edible films

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Abstract

This study investigated a novel method of photosensitizer-induced cross-linking (using riboflavin as a sensitizer) to improve the structural and physicochemical properties of gelatin-based edible films with different glycerol concentrations (25% and 50%) during different UV exposure times (2, 4 and 6h). The films' tensile strength was enhanced significantly for both glycerol concentrations with increasing UV exposure times compared to the control film, so that the highest tensile strength was observed for films with 25% glycerol and 6 h of UV exposure (25%-6h). The films' tensile strength declined and the elongation at break increased about three times when the glycerol concentration was increased to 50% with 6h exposure. The photosensitizer-induced cross-linking significantly reduced the films' solubility and permeability. The UV-treated films exhibited very good barrier properties against UV, with zero light transmission at a wavelength of 200 to 350 nm. Moreover, no toxicity was found in any of the films. In addition, Fourier transform infrared spectroscopy and differential scanning calorimetry findings revealed a good interaction between functional groups of riboflavin (as the sensitizer) and gelatin in the 25%-6h film. Therefore, this new method can be a suitable alternative to chemical methods of cross-linking biopolymers.

Keywords: Photo-oxidation, Cross-linking, Riboflavin, Gelatin edible films

1. Introduction

Synthetic or non-biodegradable plastics have negative environmental impacts that are of increasing global concern (Hosseini, Rezaei, Zandi, & Ghavi, 2013). Biodegradable packaging from renewable resources is now being used as an alternative to synthetic plastics (Costa, de Oliveira Rios, & Flôres, 2015; Hosseini, et al., 2013). The polymers derived from biomass (biopolymer), such as proteins, polysaccharides and lipids, are the main materials used to produce edible films (Costa, et al., 2015). Gelatin is a biopolymer obtained by the partial degradation of collagen from the bones, skin and tissues of animals, under acidic-alkaline conditions (Benbettaïeb, et al., 2016; Costa, et al., 2015; Jiang, Liu, Du, & Wang, 2010; Riquelme, Díaz-Calderón, Enrione, & Matiacevich, 2015).

Gelatin is an abundant, low-cost material with numerous applications and functional properties in the biomedical field (Al-Hassan & Norziah, 2012; Mohammadi, et al., 2018). It is suitable for use in the packaging industry because of its oxygen and aroma barrier properties, as well as its gelling and biodegradability properties (Divya, et al., 2018; Zhang, et al., 2010). However, it has poor mechanical properties and water resistance (Jridi, et al., 2014; Zhang, et al., 2010). Numerous procedures have been used to improve gelatin-based films' functionality, such as the use of combined films, the addition of cross-linking agents and UV exposure (Uranga, Leceta, Etxabide, Guerrero, & de la Caba, 2016). Cross-linking is possible using enzymatic (transglutaminase), chemical (formaldehyde, glutaraldehyde) or physical (UV-C irradiation) treatments (Alves, et al., 2011; Chambi & Grosso, 2006; Garavand, Rouhi, Razavi, Cacciotti, & Mohammadi, 2017).

Photo-induced cross-linking is among the more effective strategies for establishing the proper interaction between biopolymers that can occur through photo-oxidation, which is carried out in

the presence of UV, photosensitizer compounds and biopolymers. The UV exposure generates various radical compounds, such as oxygen, sensitizer and biopolymer (Cardoso, Libardi, & Skibsted, 2012; Rich, Odlyha, Cheema, Mudera, & Bozec, 2014). The reaction of these radical compounds creates cross-linking between the biopolymer chains, as well as between the biopolymer and the sensitizer agents. Several sensitizers, of which riboflavin (RF) is one, are used in photo-oxidation.

RF, the water-soluble vitamin B_2, is a yellow-green fluorescent compound present in a variety of food products (Sheraz, Kazi, Ahmed, Anwar, & Ahmad, 2014). The photoproducts formed when riboflavin pigment is decomposed by light absorption also appear when the pigment is placed under UV and visible irradiation (Castillo, Criado, Díaz, & García, 2007). Riboflavin absorbs light at wavelengths of 223, 267, 373 and 444 nm, subsequently decomposing into various products. These products could include lumiflavin (LF), lumicromum (LC), Fermi methyl flavin (FMF), carboxymethylfluanine (CMF), 2, 3-butanedione, a β-keto-acid and a diketo compound (Sheraz, et al., 2014). LF is formed under the influence of photo and alkaline pH on RF, whereas acidic or neutral conditions create LC (Sheraz, et al., 2014).

Many clinical studies have been conducted on the role of RF in binding biopolymers in tissue engineering, transplantation and corneal rejuvenation (Mencucci, et al., 2013; Tirella, Liberto, & Ahluwalia, 2012). However, no studies have been performed on the effects of photo-oxidation on the functional properties and probable toxic compounds of edible films. The objectives of this study were to examine the effects of photo-oxidation in the presence of riboflavin as a sensitizer on the physicochemical, structural and cytotoxicological properties of edible gelatin film.

2. Materials and methods

2.1. Materials

Fish gelatin (bloom number ~ 240-270, water ≤ 12%) was purchased from Biobasic (Markham, Canada). Riboflavin was purchased from the Zahravi Company (Tehran, Iran). Glycerol was purchased from Merck Chemicals Co. (Darmstadt, Germany). Collagenase II was purchased from Sigma (St. Louis, MO, USA). Penicillin/streptomycin was purchased from Gibco (Carlsbad, CA).

2.2. Preparation of the films

The preparation of gelatin films followed the procedures of Jridi et al. (2013) with modification. Firstly, a gelatin solution was prepared by mixing 4 g of gelatin with 100 ml of distilled water under continuous stirring at 45°C for 30 min. Secondly, 0.05 g riboflavin (1.25% w/w based on gelatin) was dissolved in distilled water at 55°C for 30 min until a completely monotonic solution was achieved. The riboflavin solution was slowly added to the gelatin solution and mixed at 45°C for 15 min. Thirdly, glycerol was added as a plasticizer at concentrations of 0, 25 and 50% (w/w based on gelatin), and the film-forming solutions (FFS) were stirred at 45°C for 15 min to achieve a homogeneous solution. The pH of the FFS was adjusted to 6.5 using HCl. Fifty milliliters of the solution were poured into Petri dishes of 15 cm diameter. The samples were exposed to UV light at 260 nm (HNS 8W, Italy) and a distance of 5 cm for a period of 2, 4 and 6h. Films were completely dried at 25°C and a relative humidity (RH) of 40±2% for 48h, then peeled off the plate surface. The samples were conditioned in a desiccator containing saturated magnesium nitrate (25 ±3°C - 53% RH) for 72 h before their physicochemical and structural properties were tested. Gelatin films containing 25% glycerol without both UV and riboflavin (25%-no UV & RF) and 25% glycerol with UV and without RF were considered as the control films. All treatments were made in triplicate.

2.3. Film thickness

The thickness of each sample was measured by averaging the measurements from nine positions using a micrometer (Mitutoyo Manufacturing Corporation, Tokyo, Japan) with 0.001 mm accuracy.

2.4. Moisture content

After the initial weight, the film samples (2 cm × 2 cm) were dried in an oven at 105 ± 1°C until they reached constant weight, then weighed again. The samples' moisture contents were measured in triplicate. The moisture of the films was calculated using equation (1):

$$WC = \frac{w_1 - w_2}{w_1} \times 100 \quad (1)$$

where W_1 is the initial weight and W_2 is the secondary weight after oven-drying.

2.5. Water-vapor permeability

Water-vapor permeability (WVP) was determined using the ASTM E96-05 standard method (ASTM, 2005). Circular glass test cups with an internal diameter of 4 cm and a height of 10 cm were filled with anhydrous calcium chloride (15 g). Then the cups were covered and placed in a desiccator containing saturated NaCl solution at 25°C (0% RH, 0 Pa water-vapor pressure). The water was transferred along the film surface, adsorbed by the calcium chloride. The cups were weighed at 24 h intervals over seven days. The slope of mass loss versus time was calculated with a linear regression of $r^2 \geq 0.99$. The WVP of the films was calculated using equation (2):

$$WVP = \frac{W \times X}{t \times A \times \Delta P} \quad (2)$$

where W is the weight change (g), X is the mean thickness of film (m), A is the film area (m^2), t is time (s) and ΔP is the difference of vapor pressure across the film (Pa).

2.6. Water solubility

Gelatin film samples (3 cm × 2 cm) were weighed and placed in dishes containing 50 ml of distilled water and stirred at 25°C for 24 h. Undissolved debris was filtered out using filter paper, and the paper was dried at 105°C for 1 h. The solubility of the films was calculated using equation (3):

$$\text{Solubility} = \frac{w_1 - w_2}{w_1} \times 100 \quad (3)$$

where W_1 is the initial weight and W_2 is the weight of the undissolved film after drying.

2.7. Mechanical properties

Mechanical properties were determined using ASTM D882-91 (Tongdeesoontorn, Mauer, Wongruong, Sriburi, & Rachtanapun, 2012) with a texture analyzer (SMT-20, Santam, Tehran, Iran). The films were placed in the desiccator for 48 h at 50 ± 5% RH at 25°C. The tensile strength (TS) and percentage of elongation at break (EAB) of the film samples (1 cm × 10 cm) were measured at a cross-head speed of 10 mm/min with an initial grip length of 5 cm. The tests were performed in triplicate and the average of the results was calculated for each film.

2.8. Fourier transform infrared spectroscopy measurement

To obtain the films' Fourier transform infrared (FTIR) spectroscopy spectra, each sample was placed into the spectroscope's crystal cell, which was mounted on an FTIR spectrometer (Shimadzu Irprestige-21, Japan). The FTIR spectra were obtained using an attenuated total reflection (ATR) accessory in the range of 500 to 4000 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans, and the data were controlled against a background spectrum. The rectangular film samples were placed directly onto the spectrophotometer cell.

2.9. Differential scanning calorimetry (DSC)

The film samples were conditioned in desiccators with silica gel to dehydrate them for at least three weeks. The thermal properties of the samples were assessed using a differential scanning calorimeter (DSC 822e, Mettler Toledo, Switzerland). Samples of about 10 mg were weighed and encapsulated in aluminum pans individually and heated at a rate of 10°C/min under a nitrogen flow of 10 cm^3/min at a temperature range of -20 to 150°C. An empty aluminum pan was used as a reference.

2.10. Light transmission and transparency

Initially the films were cut and placed in the test cell of a UV-visible spectrophotometer (S2100SUV, UNICO, Shanghai) that was calibrated using an empty test cell. The films' light transmission was measured in wavelengths between 200 and 800 nm. The transparency value was determined using equation (4):

$$\text{Transparency value} = \frac{-\log T_{600}}{x} \quad (4)$$

where T_{600} is the transmittance at 600 nm and x is the film thickness (mm). According to the equation, the higher the transparency value, the lower the films' transparency.

2.11. Cytotoxicity test

2.11.1. Cell culture

The viability of the human adipose tissue-derived mesenchymal stem cells (hADSCs) and NIH 3T3 cells (mouse embryonic fibroblast cell line) seeded on the gelatin membrane was determined by MTT assay. NIH 3T3 fibroblasts were obtained from the Pasteur Institute of Iran (Tehran, Iran) and expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were sub-cultured when they reached 90% confluence. NIH 3T3 in passage 3 was used in this study (Rostami, et al., 2015).

The hADSCs were isolated from human adipose tissue collected after elective liposuction surgery according to procedures approved by the Ethics Committee at Shahid Beheshti University of Medical Sciences, Tehran, Iran (Gholipourmalekabadi, et al., 2016). Briefly, the tissues were rinsed twice with phosphate-buffered saline supplemented with antibiotics and antifungals, and then treated with 0.2% collagenase II for 30 minutes at 37 ^{0}C. The samples were centrifuged and the supernatant was discarded. The cell pellet was resuspended in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin by micro-pipetting and seeded in two T-75 cm^2- cell culture flasks. The cell medium was changed every three days. After reaching 80% confluence, the cells were sub-cultured using trypsin/ethylene diamine tetra-acetic acid (EDTA). The cells of the second passage were used in this study.

2.11.2. MTT assay

Cell viability was determined based on the ability of the living cells' mitochondria to reduce the level of tetrazolium salt (MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide]). MTT assay was carried out by a slightly modified procedure described previously (Samadikuchaksaraei, et al., 2016). For this purpose, 2×10^4 cells were gently seeded on the 25%-6h treatment (1 cm × 1 cm), feed with a supplemented DMEM (described above) and incubated in a humidified atmosphere of air and 5% CO_2 at 37°C for 1, 2, 3, 7 and 14 days. After each interval, the cells were treated with 10% MTT solution for 2 h. The cells were then washed with phosphate-buffered saline and treated with dimethyl sulfoxide (DMSO). The optical density of the samples was measured with an ELISA (enzyme-linked immunosorbent assay) reader at a

wavelength of 590 nm. The cells cultured on the plastic surface of the cell culture flask served as a negative control, which was considered to be 100% cell viability.

2.12. Statistical analysis

The statistical analysis was conducted using one-way analysis of variance (ANOVA) using SPSS software (version 23.0, SPSS Inc., Chicago, USA). A Duncan's multiple range test method was employed to detect significant ($p< 0.05$) differences.

3. Results and discussion

3.1. Solubility

Due to the hydrophilic properties of proteins (which are in turn due to the presence of polar peptides), gelatin films are highly soluble in water; this is a limitation on their use for packaging applications (Mohajer, Rezaei, & Hosseini, 2017). The solubility values of film samples are showed in table 1.For the film samples at the 25% glycerol concentration, solubility was decreased by increasing UV exposure time from 2 to 6h. However, the greatest solubility reduction (30.66%, significantly lower than the control film) was observed for treatment 25%-4h. This could be due to the interaction between the functional groups in gelatin and the riboflavin as a sensitizer, which acts as a physical barrier to prevent the penetration of water into the gelatin film network. However, the values for the solubility of films for all treatments containing 25% glycerol were less than half of those found in other studies on gelatin films. The obtained quantities were lower than the results reported by Hosseini et al. (Hosseini, et al., 2013) and Núñez-Flores et al. (Núñez-Flores, et al., 2012) for gelatin films made from the skin of cold-water fish (~63.81%) and from cod skin (~92%). Moreover, values for solubility in the present

study were lower than those found for gelatin films containing cross-linking agents such as transglutaminase (Chambi, et al., 2006) and glutaraldehyde (Alves, et al., 2011).

Film solubility increased with an increase in glycerol concentration from 25% to 50%. However, the UV exposure times had significant effects on the solubility of films containing 50% gelatin. Glycerol, due to its high hydrophilicity, can lead to increased water absorption, which causes the film structure to unfold and increases the film solubility. However, the solubility of films in all treatments containing 50% glycerol were less than that found in previous studies. This difference could be due to the cross-linking effect of the phototoxidation mechanism, which caused part of the glycerol to participate in the film structure, resulting in a decrease in its hydrophilicity.

3.2. Water-vapor permeability

The WVP of edible film mainly depends on the structure of its polymers, its thickness and its molecular weight (Jridi, Souissi, et al., 2013; Li, Miao, Wu, Chen, & Zhang, 2014). Table 1 presents the degree of WVP in the photosensitizer-treated gelatin films and the control gelatin film. The results showed that the treated films had a dramatically significant relationship with WVP compared to the control film. Many protein-based edible films, particularly those made with gelatin, increase WVP due to their non-linear and complex structure and their long-chain and high-hydrophilic groups (Etxabide, Urdanpilleta, de la Caba, & Guerrero, 2016). The photosensitizer-treated gelatin films reduced the free spaces between the polymer matrix and the amount of hydrophilic amino acids on the film surface due to the different cross-links between gelatin-riboflavin-gelatin and gelatin-gelatin, resulting in a stronger film structure and less permeability by water molecules through the polymer.

Increasing the UV exposure times decreased the WVP for both glycerol concentrations. The lowest value for all treatments was found for the treatment 25%-6h (3.12). This was a lower

WVP, significantly different from the values found for gelatin films cross-linked with transglutaminase (0.066 ×10-8 g × (m × s × Pa)$^{-1}$) (Kołodziejska & Piotrowska, 2007), glutaraldehyde (0.020 × 10^{-8} g × (m × s × Pa) $^{-1}$ (Chiou, et al., 2008) and nano-chitin (8.89 × 10^{-10} g × (m × s × Pa) $^{-1}$ (Sahraee, Milani, Ghanbarzadeh, & Hamishehkar, 2017). However, the WVP was higher than that found for films containing cross-linking agents such as tannin acid (6.7 × 10^{-13} g × (m × s × Pa) $^{-1}$ and ferulic acid (5.5×10^{-13} g × (m × s × Pa) $^{-1}$ (Cao, Fu, & He, 2007). This difference can be attributed to the nature of the polymers and the type and concentrations of the cross-linking agents. The WVP was greater in all treatments at the 50% glycerol concentration relative to the 25% glycerol concentration. This trend is in line with other studies of plasticizer concentration, probably because of the increased mobility and further spaces between the polymers imparted by the plasticizer. Such results have also been confirmed in tests of the films' mechanical properties.

3.3. Mechanical properties

Mechanical properties were usually related to the films' intermolecular force and network microstructure (Table 1). The tensile strength increased significantly from 18.7 to 77.8 MPa by increasing the photo-oxidation time from 2 to 6h at the 25% glycerol concentration. Furthermore, the tensile strength of the 25%-6h treatment was about eight times greater than that of the control sample. The results show higher tensile strength for the photosensitizer-treated gelatin films in comparison to many gelatin films produced with cross-linking agents.

These findings suggest that UV in the presence of a riboflavin sensitizer increases films' tensile strength by forming gelatin-gelatin and gelatin-riboflavin-gelatin polymer cross-links. Previous studies reported different results regarding the effect of radiation on films' mechanical properties. Sionkowska et al. (2006) stated that the tensile strength was reduced due to the

breakdown of peptide bonds through increasing UV exposure time in the collagen film (380 nm). However, Benbettaïeb et al. (2016) reported that electron-beam irradiation increases the tensile strength of gelatin films. The difference in these results may be due to differences in the radiation conditions and the film-production methods.

With an increase in the concentration of glycerol from 25% to 50%, the films' TS decreased and EAB increased. For the films at the 50% glycerol concentration, the TS was enhanced by increasing the UV exposure time from 2 to 6h; the TS was significantly different from that of the control treatment.

In all photosensitizer-treated gelatin films, the EAB percentage was improved compared to the control film regardless of the duration of the UV exposure. The 25%-6h and the 50%-2h treatments showed the lowest (44.0%) and highest (154.6%) EAB values among photosensitizer-treated films, respectively. Compared to previous studies, the EAB and TS values were higher for gelatin films at the same glycerol concentration (Cerqueira, Souza, Teixeira, & Vicente, 2012; Hanani, McNamara, Roos, & Kerry, 2013). This may be due to the interaction between gelatin chains caused by the photo-oxidation mechanism.

3.4. FTIR-ATR analysis

FTIR spectroscopy was performed to evaluate the possible cross-linked interactions of films due to UV radiation. As shown in Fig. 1, the FTIR spectra of neat gelatin film indicate the characteristic absorption bands at 3275 cm^{-1}, 2927 cm^{-1}, 1631 cm^{-1}, 1539 cm^{-1} and 1234 cm^{-1}, corresponding to the NH stretching coupled with hydrogen bonding from amide-A; NH_3^+ bending and C-H stretching from amide B; C=O stretching/hydrogen bonding (coupled with COO) from amide-I; N-H bending and C-N stretching from amide-II; and C-N and N-H in-plane stretching or CH_2 vibrations of glycine from amide-III, respectively (Aewsiri, Benjakul, &

Visessanguan, 2009; Hoque, Benjakul, & Prodpran, 2011; Muyonga, Cole, & Duodu, 2004a, 2004b). According to Muyonga et al. (Muyonga, et al., 2004b), the amide I absorption band between 1600 and 1700 cm^{-1} is a useful peak for FTIR analysis of the secondary structure of proteins like gelatin. Spectra of different samples revealed significant changes among the functional groups. The peaks' intensity under UV radiation in films without a riboflavin sensitizer (25%-6h-no RF) were higher than for the control film. Moreover, UV radiation caused an increase in the wavenumber of the amide A, II and III bands, probably due to stimulation of the crosslinking reaction among functional groups of gelatin by UV rays. Contrary to these results, Sionkowska reported that solar UV radiation of collagen films resulted in the scission of hydrogen bonds and a shift of the amide A and I bands to lower wavenumbers (Sionkowska, 2006). The difference in results could be due to the type of biopolymers, the wavelength of UV rays and/or other experimental conditions.

The intensity of FTIR spectra peaks decreased with the addition of riboflavin as a photo-sensitizer and UV irradiation (treatment 25%-6h) compared to treatment 25%-6h-no RF. However, the intensity of the amide A, B, I and II bands was higher than that for the control film. These results showed that riboflavin and UV irradiation in treatment 25%-6h resulted in fewer amide bonds than treatment 25%-6h-no RF, and more amide bonds (except for amide III) than for the control film. However, the presence of the photosensitizer in UV-radiated film caused an increase in the intensity of the band at ~1276 cm^{-1} (probably related to diarylamines). Moreover, a new drastic dip at 1700-2000 cm^{-1} was formed in the presence of both the photosensitizer and glycerol in UV-radiated gelatin film. These changes could be due to photo-oxidation mechanisms and cross-linking of gelatin functional groups, especially the cross-linking of aromatic amino acids (tyrosine and phenylalanine) with the N and C-O groups of excited

riboflavin and glycerol, respectively. Fig. 2 shows the main possible interactions resulting from photo-oxidation in edible gelatin films. These results confirmed those from the examination of mechanical properties.

Plasticizer concentration is an important factor in film structure. A new method for film preparation was used in this study. Therefore, it was necessary to investigate the effects of glycerol on the films' FTIR spectra. Uranga et al. (2016) reported that the typical absorption bands of glycerol are located in the range of 800 to 1150 cm^{-1}, which is associated with the vibrations of C-C and C-O bonds. Figure 1 shows that the intensity of the main typical glycerol spectrum peaks decreased in treatment 25%-6h compared to 0%-6h, probably due to new interactions of glycerol and the formation of a new drastic dip at 1700-2000 cm^{-1}. The intensity of these peaks increased in treatment 50%-6h. Similarly, Hanani et al. (Hanani, et al., 2013) and Núñez-Flores et al. (Núñez-Flores, et al., 2013) reported an increase in the peak intensity at 1033 cm^{-1} related to the presence of glycerol; this may be due to extra interactions between the glycerol and film structure. Moreover, the wavenumber of amide A decreased and that of the II bands increased with increasing glycerol concentration. These variations probably indicate an increase in the number of chemical bonds and interactions between UV-excited glycerol (C-O) and stimulated functional groups of gelatin and/or riboflavin.

3.5. Thermal properties

The thermal properties of polymers are significant in determining their usefulness as a packaging material. It is important to study their heat stability during preparation, processing or consumption in the packaging industry (Jridi, Nasri, et al., 2013). DSC thermograms, partially verified by FTIR findings (Fig. 3), were used to investigate the cross-link reactions in the UV-treated films. In gelatin films, the melting transition temperature is associated with the helix-coil

transition during gelatin denaturation compared to the native status. The helix-coil transition revealed the disappearance of hydrogen bonds in the triple helix and the rearrangement of the gelatin skeleton (Alves, et al., 2011). Therefore, the melting enthalpy (ΔH_m) has a direct relation to the value of the triple helix in the film matrix (Chambi, et al., 2006). One of the important factors in the thermal stability of gelatin films is the amount of proline and hydroxyproline amino acids in the gelatin triple-helical structure, which is different in various references. In the current study, the melting point was higher for the UV-treated films than for the control sample. This may be due to the formation of further intramolecular and intermolecular hydrogen and covalent bonds by exciting the functional groups of riboflavin and gelatin during photo-oxidation. The melting point obtained in treatments under UV radiation was higher than the result obtained by Ma et al. (Ma, et al., 2012). The melting point was elevated by increasing UV exposure time in both glycerol concentrations to 108.22ºC for the 25%-6h treatment and 73.25ºC for the 50%-6h treatment. The melting point was decreased by increasing the glycerol concentrations from 25% to 50%, possibly due to the partial inhibitory effect of glycerol on the formation of protein structures. Generally, the results of mechanical properties, DSC and FTIR showed the proper interaction between gelatin and riboflavin.

3.6. Light transmission and transparency

The amount of light transmission in food packing is considered to be a key factor in maintaining the quality of coated foodstuffs. Light transmission (especially in the UV wavelengths) causes food discoloration, reduced nutritional value, auto-oxidation and other undesirable chemical reactions in packaged foods (Hosseini, et al., 2013). The results of the current study showed that the amount of UV light transmission was zero in all UV-treated samples at the wavelength range of 200 to 350 nm. Previous studies on gelatin films have reported that the amount of light

transmission up to 350 nm was less than 50%. The main reason for this great difference can be UV absorption through riboflavin amide rings in the gelatin films' composition. However, it has been found that less light is transmitted through gelatin films due to the presence of cyclic amino acids (tyrosine and tryptophan) than through other protein films (Mohammadi, et al., 2018). The amount of light transmission was enhanced significantly in the current study by increasing the wavelength from 400 to 800 nm. One of the other optical features of edible films is their transparency index, with a higher transparency index indicating greater opacity. As shown in Table 2, there was no significant difference in transparency index between any of the photosensitizer-treated gelatin films. The transparency index depends on the internal structure of the films, indicating proper interaction between polymers. In general, the photosensitizer-treated gelatin films were an effective barrier against UV light, which is a crucial characteristic in food packaging.

3.7. Cytotoxicity

According to previous studies, chemical cross-linking agents such as glutaraldehyde, boric acid, ethylene glycol diglycidyl ether, ammonium zirconium carbonate and H_2SO_4 can improve the mechanical properties and water resistance of edible films (Garavand, et al., 2017). Nevertheless, residues of unreacted cross-linking agents and their interaction between polymers could be a source of toxicity. This study has examined a new method for the formation of cross-links between biopolymers using UV light and a sensitizing agent in gelatin films; therefore it was essential to investigate the toxicity of the films. Fig. 4 shows the cytotoxicity results for the gelatin films in the current study. Light-microscope photographs of the hADSCs and NIH 3T3 are shown in Fig. 4A, and the MTT results are shown in Fig. 4B. The microscopic results in passage 3 proved the presence of the spindly cells of the hADSCs and NIH 3T3. The MTT assay

measures the cell's capacity to reduce MTT, and is therefore a direct measurement of cell viability. Only cells that are metabolically healthy and normal can convert tetrazolium salt into purple crystals (Draye, et al., 1998). The more active and numerous the cells, the more intense the color created (Manna, et al., 2015). MTT results showed that UV-treated films (25%-6h, 0.05 g riboflavin) did not change the average viability of either cell types during incubation for 14 days ($p>0.05$).Therefore, films produced by photo-oxidation were non-toxic and biocompatible with mesenchymal and fibroblast cells.

4. Conclusion

The results of this study showed that the mechanical properties of gelatin-based films were significantly improved by increasing the UV exposure time up to 6h, and the films' WVP and solubility were decreased. Following an increase in glycerol concentration from 25% to 50%, solubility, WVP and EAB were increased and the film TS was decreased. The physicochemical indices of all films showed remarkable improvement compared to the control film, which indicated that this new method incorporated a percentage of glycerol in the film structure and reduced its barrier role. The structural characteristics (as measured by FTIR and DSC) and the physicochemical properties of the films showed that the 25%-6h and 50%-6h films among all treatments were suitable films, depending on their various applications. This new method, which enhances the interaction between polymers by forming cross-links between different functional groups, requires further research into the interaction between polymers and various natural sensitizers, as their lack of toxicity suggests that they can serve as alternatives to other chemical cross-linking methods.

5. Acknowledgments

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Fig. 1. FTIR spectra of films with different concentrations of glycerol.

Fig. 2. The main possible interactions resulting from photo-oxidation mechanism in gelatin edible films

Fig. 3. DSC profiles of gelatin films with different concentrations of glycerol at different times of radiation.

Fig. 4. (A) Light microscope photographs of the hADSCs and NIH 3T3 cells attached on the plastic surface of cell culture flask. (B) MTT results of hADSCs and NIH 3T3 cells after 1, 2, 3, 7 and 14 days incubation with gelatin membrane. Cell viability of negative control (the cells cultured in supplemented DMEM without collagen membrane) was considered as 100% cell viability.

Table1 Moisture content, film solubility, water vapor permeability, Tensile strength (TS), elongation at break (EAB) of gelatin films.

Films	Thickness (μm)	Moisture content (%)	Water solubility (%)	Water vapor permeability (10^{-12} g × (m × s × Pa) $^{-1}$)	TS (MPa)	EAB (%)
25%- C	113.6±1.9e	19.7±0.8a	90.2±0.8a	74.3±0.1a	8.6±0.3j	3.5±1.6f
25%-2h	123.8±1.6b	8.9±0.3e	37.8±0.1d	5.8±0.3d	18.1±0.1e	62.6±2.2d
25%-4h	119.4±1.1c	13.2±0.6c	30.7±0.2f	3.7±0.3f	23.1±0.1b	74.1±2.0c
25%-6h	115.9±0.9d	13.1±0.4c	32.4±0.5e	3.0±0.1f	77.8±0.3a	44.0±1.9e
50%-2h	127.3±0.8a	10.5±0.9d	40.8±0.6c	9.8±0.2b	14.9±0.1f	154.5±3.4a
50%-4h	125.8±2.1ab	10.9±0.3d	43.1±0.3b	6.1±0.1c	19.4±0.4d	125.7±3.7b
50%-6h	123.2±0.6b	15.2±0.7b	44.5±0.9b	5.0±0.1e	21.8±0.2c	147.3±1.4a

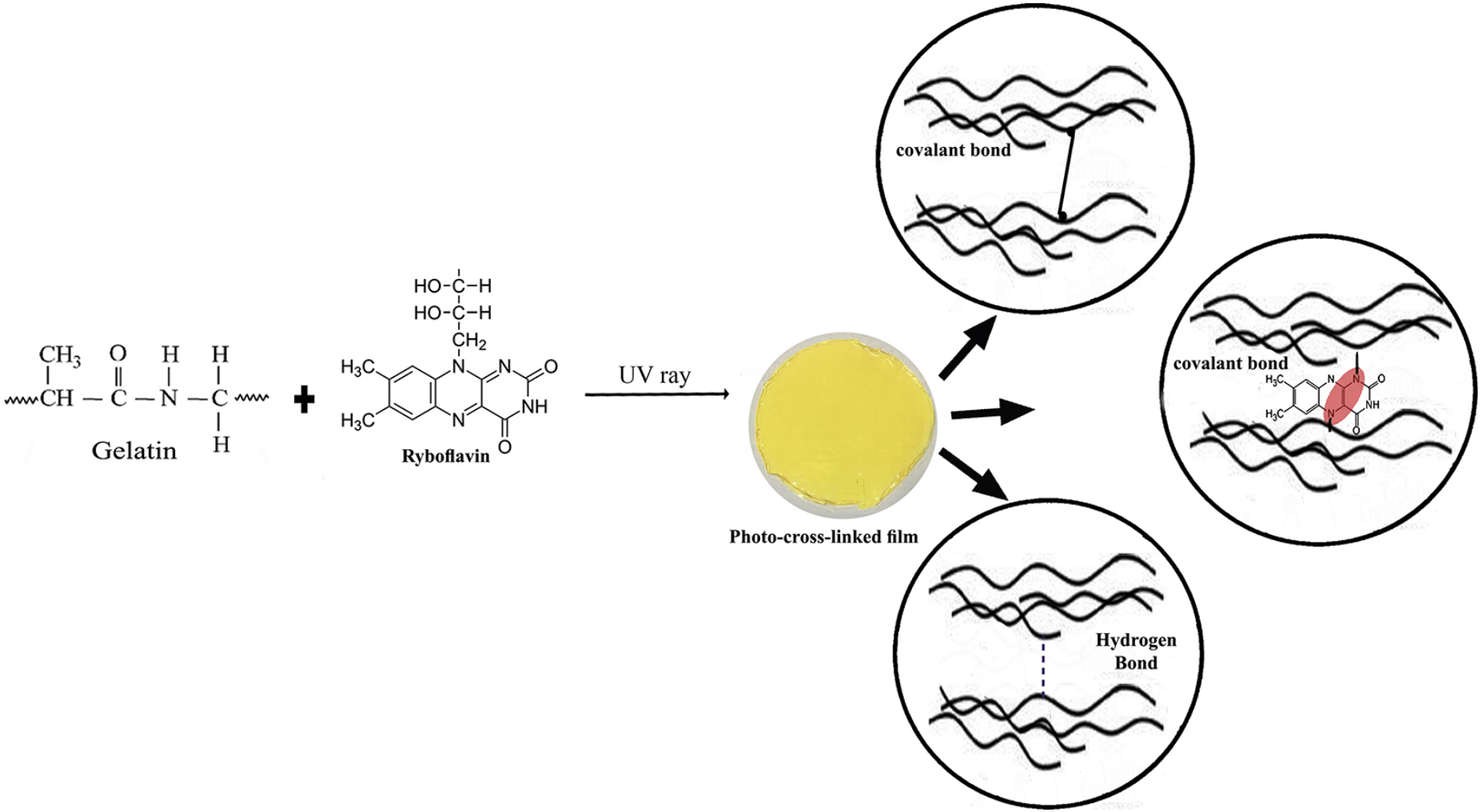
Reported values for each film are means ± standard deviation. Values means followed by the same letter are not significantly ($p > 0.05$) different according Duncan's multiple range test.

Table 2 Light transmission (%) and transparency of films obtained with different ratios of gelatin films.

sample			Wavelength (nm)							Transparency
	200	260	280	350	400	500	600	700	800	
25%-2h	0	0	0	0	0.7	55.4	79.7	85.6	87.8	0.0044
25%-4h	0	0	0	0	0.8	52.2	81.8	85.5	87.5	0.0046
25%-6h	0	0	0	0	0.8	59.1	81.2	85.3	86.9	0.0048
50%-2h	0	0	0	0	0.2	43.3	83.3	88.2	89	0.0040
50%-4h	0	0	0	0	0.4	50.2	82.6	87.2	89.1	0.0040
50%-6h	0	0	0	0	0.6	60.2	81.9	86.5	88.1	0.0047
25%-no uv & RF	0.0	6.3	8.6	58.2	65.3	73.3	84.5	86.1	90.7	0.0047

Highlights

- The UV ray in the presence of riboflavin sensitizer significantly reduced the solubility and WVP of the gelatin films.
- The highest tensile strength was observed in the treatment 25%-6h in the presence of riboflavin sensitizer.
- The structural analysis revealed a good interaction between functional groups of riboflavin and gelatin in the 25%-6h film.
- The amount of UV light transmission was zero in all photosensitizer-treated gelatin films.



Graphics Abstract

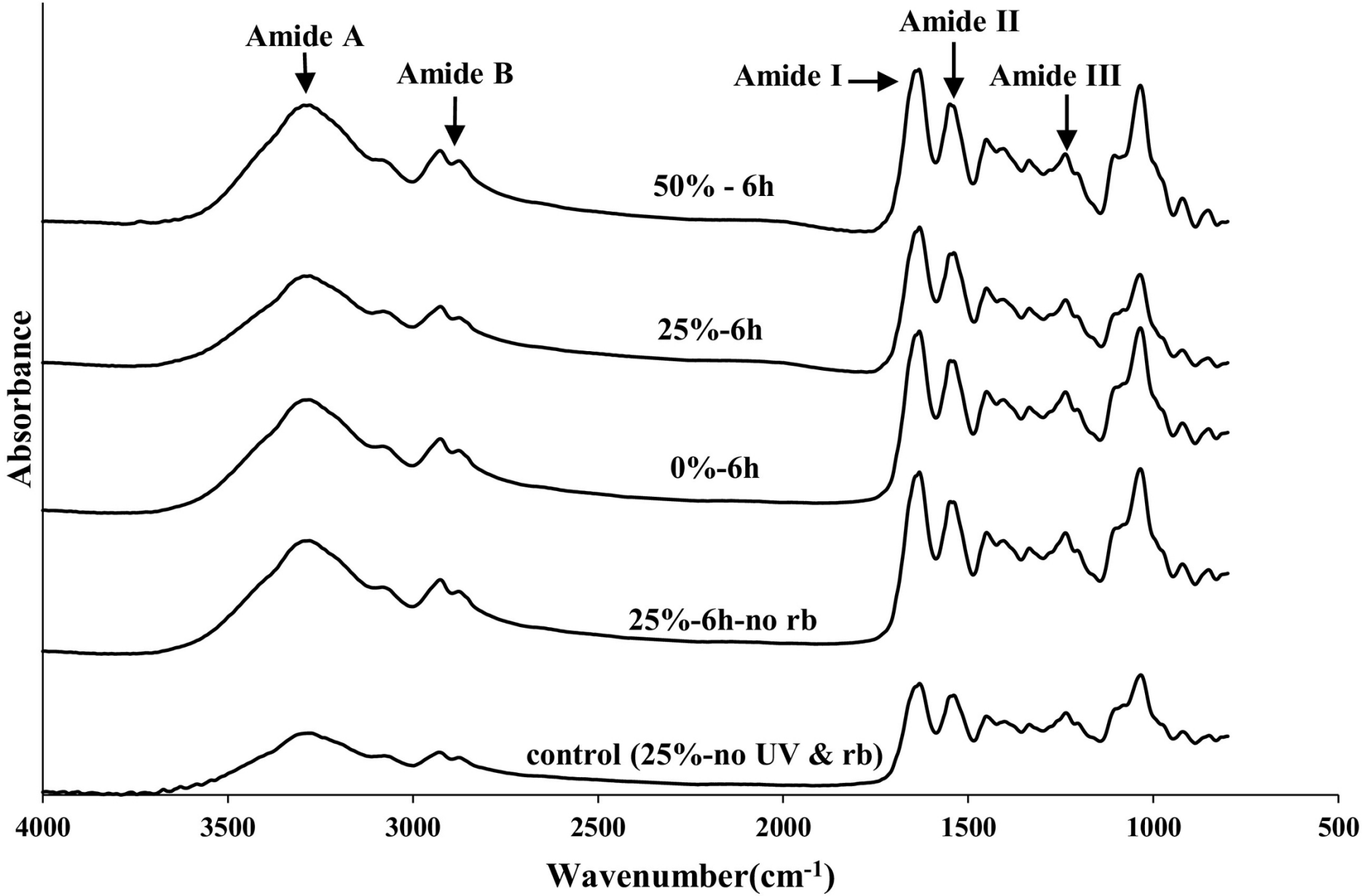

Amide A
Amide B
Amide I
Amide II
Amide III
50% - 6h
25%-6h
0%-6h
25%-6h-no rb
control (25%-no UV & rb)
Absorbance
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber(cm-1)

Figure 1

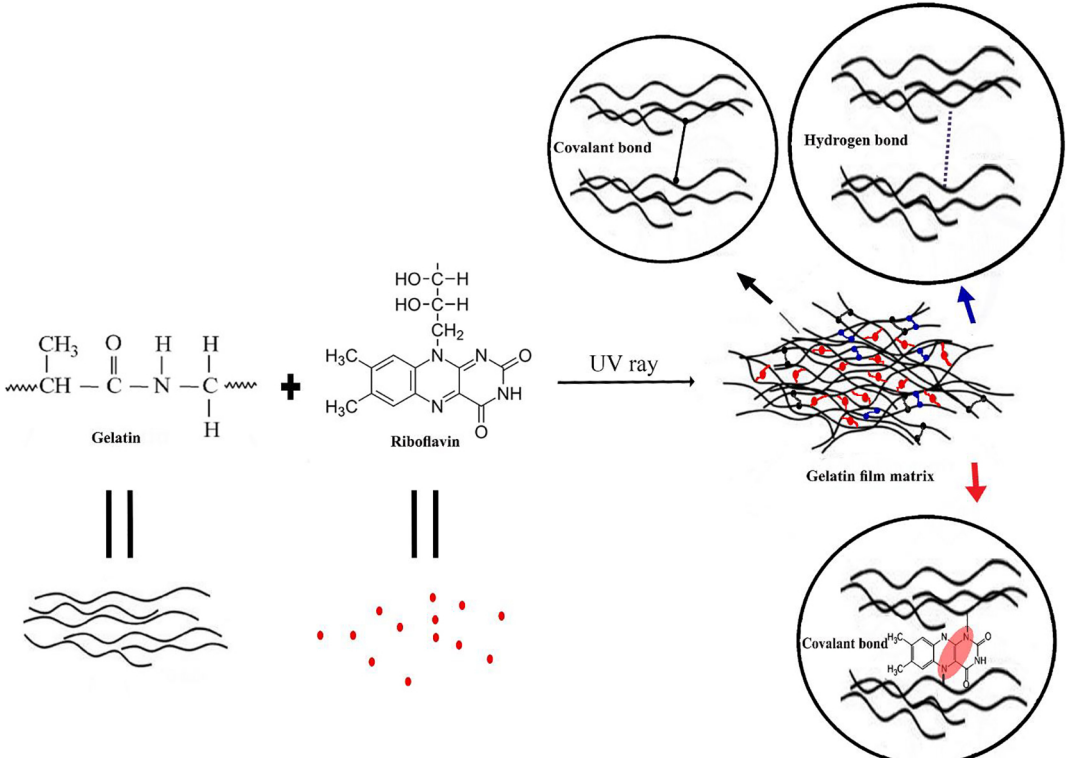

Covalant bond
Hydrogen bond
Gelatin
Riboflavin
UV ray
Gelatin film matrix
Covalant bond

Figure 2

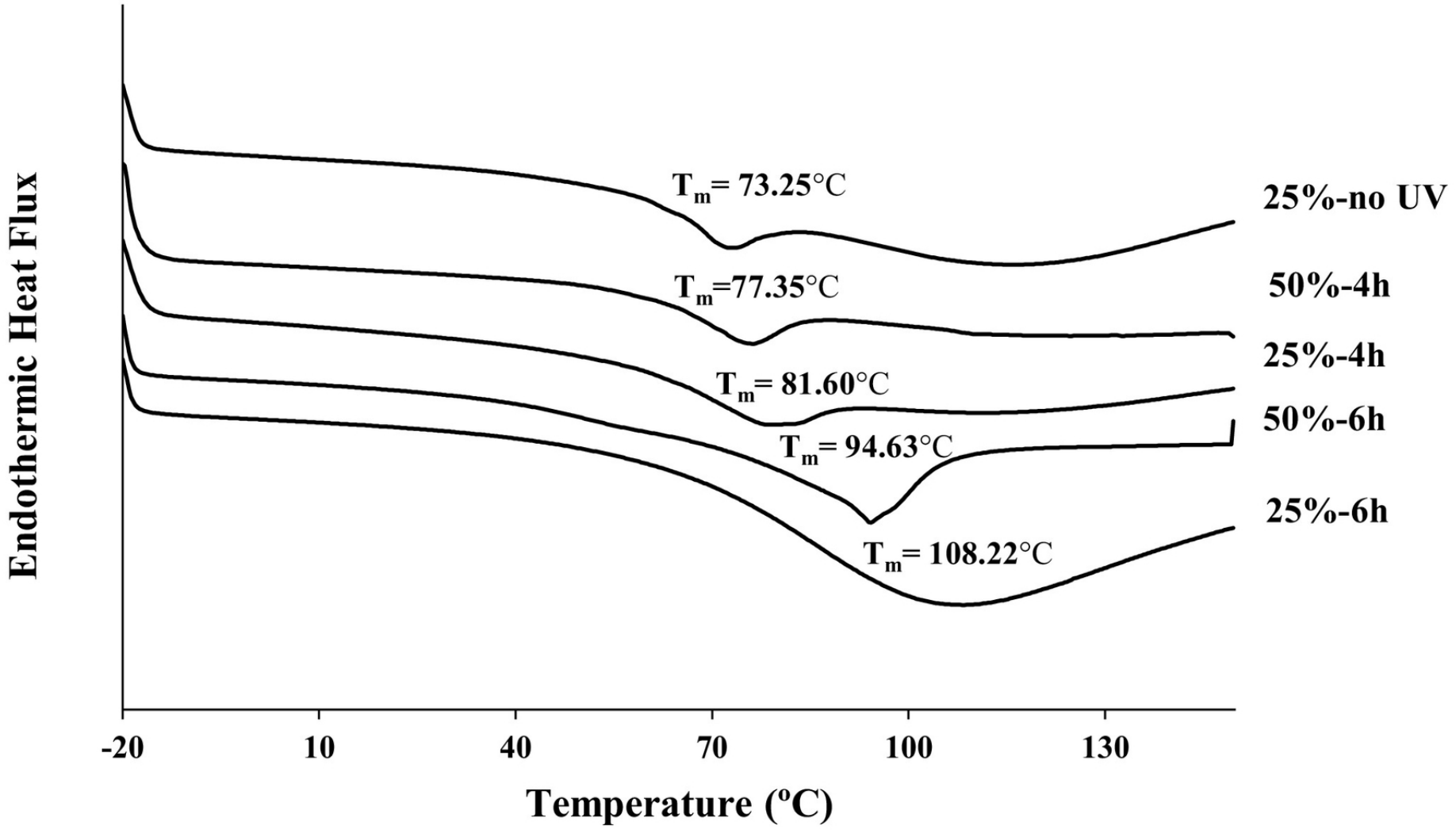

Endothermic Heat Flux
T_m= 73.25°C
25%-no UV
T_m=77.35°C
50%-4h
25%-4h
T_m= 81.60°C
50%-6h
T_m= 94.63°C
25%-6h
T_m= 108.22°C
-20
10
40
70
100
130
Temperature (°C)

Figure 3

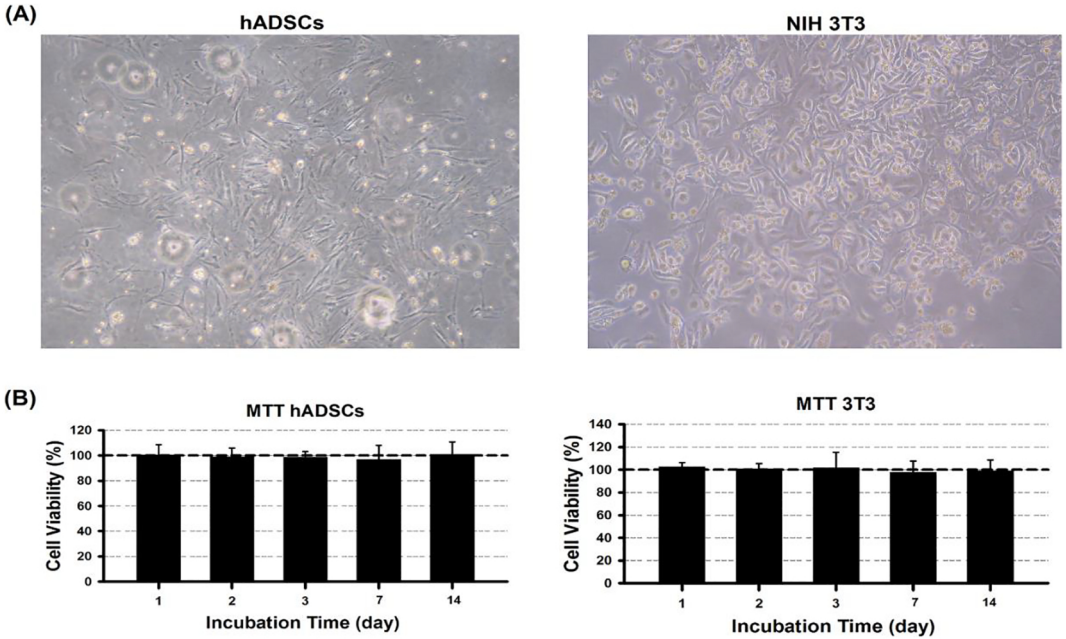

(A)
hADSCs
NIH 3T3
(B)
MTT hADSCs
Cell Viability (%)
120
100
80
60
40
20
0
1
2
3
7
14
Incubation Time (day)
MTT 3T3
Cell Viability (%)
140
120
100
80
60
40
20
0
1
2
3
7
14
Incubation Time (day)

Figure 4